Heterogeneous Response for a Mammalian Hepadnavirus Infection to Acyclovir: Drug-Arrested Intermediates of Minus-Strand Viral DNA Synthesis Are Enveloped and Secreted From Infected Cells as Virion-Like Particles

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Three woodchucks infected persistently with the woodchuck hepatitis virus (WHV) were treated with acyclovir (ACV) to investigate the effect of inhibiting viral DNA synthesis upon the replication of an orthohepadnavirus in vivo. Normal viraemia was reduced during the treatment period in all three animals, but each responded with a distinct serum phenotype. In the most provocative case, the profile of the WHV DNAs in both the liver and serum provided a simple and novel description of the orthohepadnaviral infection for this ACV protocol. The pre-drug viraemia was rapidly cleared from the serum and replaced by virion-like particles containing predominantly minus-strand WHV DNAs. These serum DNA species had the character of replicative intermediates arrested in their elongation by ACV-mediated chain termination and were contained in particles with a buoyant density in CsCl essentially identical with virions. However, in infected hepatocytes, initiation of reverse transcription within newly formed core particles was not inhibited by the ACV treatment. Instead, an heterogeneous array of minus-strand DNAs were synthesised, each presumed to be truncated by the incorporation of one molecule of ACV monophosphate. An approximately normal level of core particles was present in the liver of this woodchuck after 26 days of the ACV protocol; excess drug-arrested nucleocapsids were steadily removed throughout the dosing period upon their envelopment and secretion as virion-like particles into the circulation. These data suggest that plus-strand DNA synthesis may not be absolutely required prior to secretion of virus from the infected cell. ***J Med Virol 51:6–16, 1997.***

KEY WORDS: hepatitis B virus; morphogenesis; DNA replication; antiviral

INTRODUCTION

Dissection of the multiple steps involved in the replication cycles of viruses is often achieved by genetic analysis. Mutant genomes have been exploited successfully for this purpose in hepadnaviruses [Chang et al., 1987; Schlicht et al., 1987; Yaginuma et al., 1987], but the approach remains complicated by the overlapping nature of their genes and the parsimonious use of the viral DNA that so characterizes the members of this virus family [for a review, see Ganem and Varmus, 1987]. An additional strategy is to perturb the replication processes biochemically, usually with metabolic inhibitors, and obtain insight from the character of the modified, and perhaps abortive, infection. In the absence of suitable viral mutants, we have used the chemical approach to specifically interrupt viral DNA synthesis for an orthohepadnavirus in vivo. Several limited trials were conducted with the woodchuck hepatitis virus (WHV) system to permit preliminary evaluations of candidate inhibitors, including the one described in this report, acyclovir (ACV).

ACV is an analogue of deoxyguanosine and is a well-characterised and specific inhibitor of DNA synthesis for members of the herpes virus family [Elion, 1993]. When tested in the duck hepatitis B virus (DHBV) system, administration of ACV to infected ducks produced an immediate and stable reduction of viraemia, whether administered orally [Tsiquaye et al., 1986] or by intraperitoneal injection (Cullen, J.M., Tencza, M.G., and Newbold, J.E., unpublished data). Now recognized as

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the typical response to inhibitors that are nucleoside analogues, the serum titre of DHBV rebounded immediately when ACV dosing ceased. Moreover, as expected, ACV triphosphate was indicated as the active inhibitor in vivo. This inference was derived from the endogenous DNA polymerase reaction associated with both DHBV liver cores and WHV virions. In these in vitro reactions, ACV triphosphate was equally effective at blocking the elongation of plus- and minus-strand DHBV DNAs as well as plus-strand WHV DNA (Newbold, J.E. unpublished data). Although ACV is very well tolerated in mammals over long treatment regimens, and is evidently active against DHBV in ducks, its phosphorylation by host enzymes in mammalian liver cells was generally considered too low to achieve significant inhibition of a sensitive DNA polymerase. However, since the DHBV and WHV polymerases show essentially identical inhibition by ACV triphosphate in vitro, ACV was nevertheless tested in woodchucks in vivo as a potential inhibitor of the WHV DNA polymerase.

The hepadnaviral genome is replicated in infected cells by a scheme that involves reverse transcription and utilizes the virus-coded DNA polymerase or reverse transcriptase [Summers and Mason, 1982]. For orthohepadnaviruses, the virion DNA is a partially double-stranded, circular molecule. Upon entry into a sensitive, permissive cell, this open circular (OC) DNA is converted into a covalently closed circular (CCC) duplex molecule that is present as a viral minichromosome in the nucleus [Newbold et al., 1995]. In the stable viral carrier state, a regulated population of 20–50 CCC DNA molecules forms in each infected cell [Miller and Robinson, 1984; Summers et al.,1991]. These nucleoprotein complexes act as the sole templates for the full programme of hepadnaviral transcription. One specialized transcript, termed the pregenome, fills a dual role in the infection process. In addition to its essential function in viral protein synthesis, this terminally redundant, 1.1 genome-sized RNA is encapsidated into cytoplasmic nucleocapsids within which each molecule of pregenome RNA is converted by reverse transcription into virion DNA [Summers and Mason, 1982]. As a first step, minus-strand DNA synthesis is primed by the terminal protein domain of the viral DNA polymerase [Zoulim and Seeger, 1994; Weber et al., 1994] and, as the nascent strand is elongated, is accompanied by the progressive degradation of the pregenome template. This latter reaction is catalysed by an RNase H that is believed to be another domain of the viral DNA polymerase [Radziwill et al., 1990]. The full-length minus-strand DNA then serves as the template for plus-strand DNA synthesis, primed by an RNA oligomer derived from the 5′ terminus of the pregenome [Lien et al,. 1986]. For virion DNA in WHV, the plus-strand ranges from 25 to 60% complete; for hepatitis B virus (HBV) the values are very similar, whereas for DHBV most virion DNAs contain a completed plus-strand. The noticeably different populations of viral DNAs present in serum virions and their precursors, the liver core particles, have led to the hypothesis that in all hepadnaviruses envelopment of nucleocapsids prior to their secretion as virions from the infected hepatocyte requires the appearance of a morphogenetic signal at the surface of the viral nucleocapsid; the nature of the signal was not formulated but necessarily coupled to a stage in plus-strand DNA synthesis [Summers and Mason, 1982].

In this study we wanted to block synthesis of WHV DNA inside nucleocapsids, in both minus- and plus-strands, in the liver cells of the persistently infected woodchucks. We expected that if the protocol worked, the outcome would parallel the experience in DHBV-infected ducks, result in a uniform and marked reduction in viraemia, and permit, in subsequent studies, a thorough analysis of the drug-aborted infection in the woodchuck livers. However, the WHV-infected woodchucks did not show a uniform response on this protocol but were instead unexpectedly heterogeneous.

MATERIALS AND METHODS

Woodchucks

The five woodchucks used in this study were trapped humanely in Pennsylvania and New Jersey. They were housed and maintained in animal facilities of the Penrose Research Laboratories at the Philadelphia Zoo; they had naturally acquired infection for WHV and were persistently viraemic for more than a 6-month period of quarantine. Woodchuck CW657 died suddenly after the quarantine from a ruptured aortic aneurysm. The liver of CW657 was promptly removed and stored at −70°C for subsequent analysis; autopsy revealed also that CW657 had a mild portal hepatitis. CW612 also died suddenly after 26 days on the ACV protocol; this liver was similarly removed and stored for later study. The cause of death for CW612 was ascribed to peritonitis, probably pursuant to the pre-trial biopsy procedure; a fatty liver was also noted in the autopsy report for CW612.

ACV Protocol

The four woodchucks enrolled in this trial were initially monitored by needle biopsy of the liver before treatments began. All animals displayed a mild portal hepatitis. Three woodchucks (CW515, CW601, and CW612) received ACV at 15 mg/kg body weight, administered every 12 hr by peritoneal injection for 6 weeks. The fourth woodchuck (CW605) received sham injections of saline on the same schedule. Blood samples were drawn weekly, or less frequently, from the four woodchucks to provide sera for the analysis of viraemia and antigenaemia; sera were stored at −70°C.

Very similar small trials were also conducted in other woodchucks to evaluate azidothymidine (AZT) and human lymphoblastoid interferon; neither compound induced any reduction in viraemia in the treated woodchucks. All procedures involving the woodchucks in these protocols were reviewed and approved by the Animal Use Committee of the Penrose Research Laboratories.

Endogenous Polymerase Reaction

Serum was assayed for endogenous DNA polymerase activity (EPA) by mixing 2 μl with an equal volume of

0.2% Triton X-100 in 2× core buffer (0.02 M Tris · HCl [pH 7.4], 50 mM NaCl, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.01% Triton X-100) and allowed to stand at ambient temperature for 5 min. The treated serum was then diluted to 44 μl with core buffer and incubated at 37°C for 1 hr in 10 mM $MgCl_2$; 0.05 mM each dATP, dCTP, dGTP, and 0.95 μCi of [^{32}P]-dTTP (800 Ci/mmol). The sample was then digested with proteinase K at 1 mg/ml in 1% sodium dodecyl sulfate (SDS) and 0.05 mM EDTA for 1 hr at 37°C. The endogenously radiolabeled WHV DNA was analysed by electrophoresis in a 1% agarose gel [Oberhaus and Newbold, 1993]. No interference or inhibition in the EPA assay was detected in the sera drawn from the three woodchucks during the ACV dosing period; this was assessed in mixing experiments using both pre- and post-drug sera as well as samples from the sham control CW605.

Ultracentrifugal Banding of Virions in CsCl

Serum (0.1 ml) was mixed in buffer containing 10 mM Tris · HCl (pH 7.4), 0.5 mM EDTA, 75 mM NaCl, and 0.4 g/ml CsCl, and centrifuged at 48,000 rpm for 48 hr at 4°C in a Beckman SW56 rotor. Fractions (0.1 ml) were collected from the gradient by bottom puncture and stored at 4°C.

DNA Extraction From Sera and CsCl Fractions

Serum samples (50 μl) or gradient fractions (0.1 ml) were incubated in 0.02 M Tris · HCl (pH 7.8), 10 mM EDTA, 1% SDS, and 50 μg/ml proteinase K in a total volume of 0.5 ml at 37°C for 3 hr. The digests were extracted with phenol:chloroform:isoamyl alcohol, sodium acetate was added to 0.3 M, 50 μg of tRNA was added as carrier, and the nucleic acids were precipitated by the addition of two volumes of 100% ethanol. The precipitated nucleic acids were collected by centrifugation, dried, and redissolved in TE (10 mM Tris · HCl, 1 mM EDTA [pH 7.6].

DNA Extraction From Woodchuck Liver Tissue

a. Standard Hirt protocol [Hirt, 1967]. A 1.0 g portion of liver was dispersed in 10 ml of cold buffer (15 mM NaCl, 5 mM EDTA, 10 mM Tris · HCl [pH 7.5]) in a Dounce homogenizer. SDS was added to 1%; NaCl was added to 1.0 M, and the mixture was set at 4°C to precipitate overnight. The sample was centrifuged at 17,000 rpm at 4°C for 45 min in the Sorvall SS34 rotor. The standard Hirt supernatant fraction was decanted and extracted with phenol buffered with 10 mM Tris · HCl (pH 7.6), and the nucleic acids were precipitated by mixing with two volumes of 100% ethanol.

b. Modified Hirt protocol [Hirt, 1967]. After dispersal of liver tissue as per protocol a, SDS was added to 1% and the extract was additionally digested with 5 mg proteinase K for 3 hr at 37°C. The procedure then followed that for protocol a. The modified Hirt supernatant fraction was extracted with the standard mixture of phenol:chloroform:isoamyl alcohol, and the nucleic acids were recovered by ethanol precipitation as before. The nucleic acid precipitates obtained by both protocols were collected by centrifugation, dried, and dissolved in TE.

WHV Riboprobes

The plasmid constructs used to prepare plus-strand (pGEM WHV 7–16) riboprobes were derived in our laboratory by H. Shi by subcloning a single copy of the complete WHV genome, strain 1 [Galibert et al., 1982] into the Eco R1 site of plasmid pGEM2 into both orientations. [^{32}P]-labeled riboprobes were synthesised as previously described [Melton et al. 1984].

Analysis of WHV DNAs

DNA samples were analysed by the Southern blot procedure as previously described [Tencza and Newbold, 1995; Oberhaus and Newbold, 1995]. To recover and detect the WHV supercoiled (SC) DNA species in the Southern analysis, it was essential to soak the agarose gels in 50 mM sodium acetate (pH 4.2) for 0.5 hr at room temperature and then in fresh solution for 0.5 hr at 50°C. The soaks at pH 4.2 cause depurination in the DNA and thereby produce nicks into all SC DNA molecules (converting them to OC DNAs). Without this preliminary depurination, the SC DNAs renature to their duplex form after the alkaline denaturation and neutralization in the gel and are not detected after the Southern transfer to nylon membrane. Quantification of the amount of [^{32}P]-labeled WHV riboprobe that hybridised to the test sample was performed by a radioanalytic imaging system (AMBIS Systems) according to the manufacturer's instructions.

WHV Surface Antigen Assay

This was performed using the AUSRIA kit (Abbott Laboratories, N. Chicago, IL) for detecting hepatitis B virus surface antigen. Serial dilutions of the woodchuck sera were used in the assay to optimize the quantification.

RESULTS

Inhibition of Viraemia in Woodchucks With ACV

This trial was undertaken to assess the affects of ACV treatment on viraemia in three woodchucks persistently infected with WHV. The drug was administered for 40 days at 12-hr intervals by peritoneal injection (15 mg ACV/kg body weight); the dose was chosen to be precisely comparable to regimens reported for, and well tolerated by, patients infected with HBV. Viraemia was monitored in serum samples prepared weekly throughout the trial and assayed by two different methods. The EPA detects nucleocapsids that contain an active viral DNA polymerase coupled with a functional endogenous primer-template; viral DNA with characteristic mobility was radiolabelled in the EPA and revealed after deproteinization, agarose gel electrophoresis, and autoradiography. Minus-strand WHV DNA, also present in serum only in viral nucleocapsids, was detected by Southern blot analysis using a plus-strand WHV riboprobe; this assay can detect WHV DNA irrespective of its functionality as either a template or

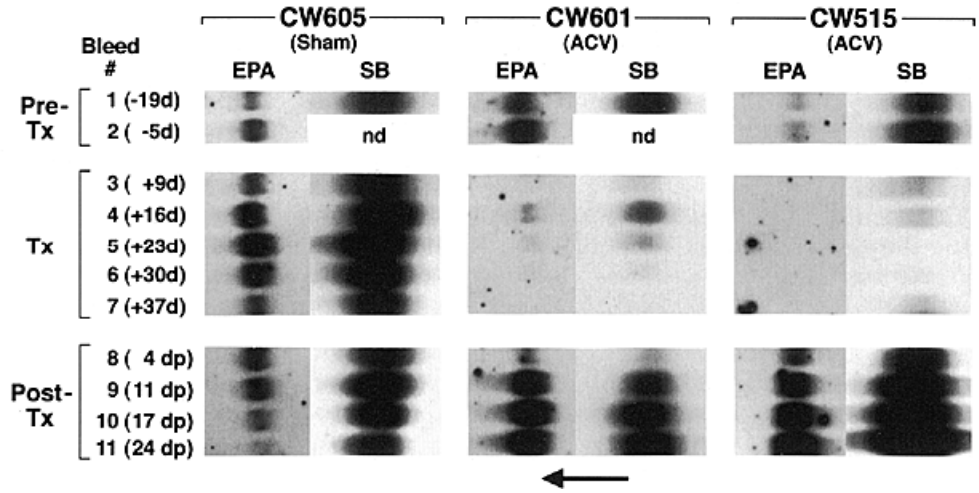

Fig. 1. Endogenous polymerase activity (EPA) and Southern blot (SB) analysis of sera from woodchucks CW605, CW601, and CW515. Eleven serial sera were obtained from each of the three woodchucks; they included two pre-treatment (pre-Tx, bleeds 1 and 2), five during treatment (Tx, bleeds 3–7), and four post-treatment (post-Tx, bleeds 8–11). ACV dosing in CW601 and CW515 commenced on day 0 and continued for 40 days (viz, 0 d to 40 d); CW605 received sham doses of saline as a control animal. Two sera (designated nd) were not evaluated by SB due to insufficient sample. For both assays the direction of electrophoresis is marked by the arrow; sections of these gels containing no signal have been cropped from this composite display.

primer. Both assays ultimately detect WHV DNA in viraemic serum as an heterogeneous population of relaxed circular DNA molecules comprising homogenous, completed minus-strand DNA base-paired to nascent plus-strand DNA of variable length. Figure 1 shows the serum profiles of these viral markers for ACV-treated woodchucks CW601 and CW515, and for one sham-treated control, CW605. The hybridisation data in Figure 1 were analysed further by calibrated phosphoimaging and converted to genome equivalents (Table I). Prior to the onset of ACV administration (bleeds 1 and 2) viraemias ranged in serum titre from 1.5×10^9 to 5.4×10^9 virions per ml. As expected, CW605, the control woodchuck that received saline injections (in lieu of ACV) during the treatment period, maintained a persistent viraemia throughout the study; the serum titre varied over a 17-fold range from 1.5×10^9 (bleed 1) to 2.3×10^{10} (bleed 5) virions per ml. Also, the heterogeneous array of WHV DNAs present in each viraemic serum from this animal displayed minor variations in both the range and distribution of their electrophoretic mobility. These differences, also noted in the samples from the other woodchucks, presumably reflected corresponding heterogeneities in the elongation and prevalence of the nascent plus-strand DNAs. The subtle changes in viral titre and extent of plus-strand DNA synthesis probably result from normal physiological variations that occur in each woodchuck.

During the treatment period, the woodchucks receiving ACV showed a marked reduction in viraemia, although the response was different in each case. For CW601, the level of circulating virus was appreciably lowered in the first bleed of the drug phase (Fig. 1, bleed 3). However, viraemia was elevated again in the next bleed and appeared to steadily decline to the background levels in the subsequent four bleeds. Four days

TABLE I. Viral Genome Equivalent/ml Woodchuck Serum

Bleed number	CW605 (sham)	CW601 (ACV)	CW515 (ACV)
1 (−19d)[a]	1.5×10^9	5.4×10^9	1.6×10^9
2 (−5d)	nd[b]	nd	2.9×10^9
3 (+9d)	1.1×10^{10}	4.9×10^8	3.6×10^8
4 (+16d)	2.0×10^{10}	1.4×10^9	2.5×10^8
5 (+23d)	2.3×10^{10}	4.9×10^8	2.6×10^7
6 (+30d)	1.6×10^{10}	1.5×10^8	4.1×10^7
7 (+37d)	9.3×10^9	5.9×10^7	4.7×10^8
8 (4dp)	7.8×10^9	8.5×10^8	6.6×10^9
9 (11dp)	1.2×10^{10}	4.0×10^{10}	1.5×10^{10}
10 (17dp)	1.0×10^{10}	7.1×10^9	2.5×10^{10}
11 (24dp)	5.5×10^9	8.7×10^9	4.9×10^{10}

[a]"−xd" denotes x days *prior* to the onset of drug treatment. "+xd" denotes x days *after* the onset of drug treatment. "xdp" denotes x days *after* the last drug treatment.
[b]"nd" indicates that this assay was not determined.

after ACV treatment ceased, a low titre of virus was again detected (Fig. 1, bleed 8). Viraemia in CW601 then rebounded to its highest level, 15-fold the pre-treatment value (Table I, bleeds 1 and 11); this elevated titre was nevertheless still lower than the highest titres measured in the control woodchuck CW605. Even though viraemia was clearly inhibited throughout most of the drug treatment phase in CW601, the reappearance of WHV to almost normal titre after 16 days of ACV treatment (Fig. 1, bleed 4) indicated that this antiviral was at the margin of its efficacy in this protocol. The second drug-treated woodchuck, CW515, responded differently to ACV than CW601. During the treatment phase, EPA was not detected, whereas WHV DNA was found at reduced levels only in the first two serum samples (Fig. 1, bleeds 3 and 4). CW515 was also different in the post-drug period when viraemia rapidly rose to the highest titre (4.9×10^{10} virions per ml) measured in this study (Fig. 1; Table I); this level of virus

represented a 30-fold increase over the pre-treatment viraemia. Bleed 11 from CW515 was not only outside the range of virus titres measured for CW605, the sham-treated control, but was the highest serum EPA detected in this laboratory from a data base of determinations from more than 300 different naturally infected woodchucks. Additional sera were obtained from CW601 and CW515, 1 month subsequent to bleed 11. In both cases, the elevated viraemias had declined to approximate the pre-drug titres (data not shown). The EPA and Southern blot assays yielded closely concordant profiles in Figure 1; the latter method was more sensitive in part because it used a 25-fold greater volume of serum. The data for CW601 and CW515 suggested that ACV treatment greatly reduced the level of WHV DNA in the serum, but that when present the DNA served as a functional primer-template and was always accompanied by active viral DNA polymerase.

In addition to CW601 and CW515, a third ACV-treated woodchuck, CW612, was included in the study. This animal died suddenly after 26 days of ACV dosing; death was ascribed to peritonitis and not related to the drug treatment (see Materials and Methods). The serum profiles of the viral markers for CW612 are displayed in Figure 2; three serum samples, corresponding to bleeds 3, 4, and 5 in Figure 1, were obtained during the drug treatment phase. Similar to the response in CW515, ACV treatment rapidly reduced the EPA to background level in CW612 (Fig. 2A). Only WHV DNA with the electrophoretic mobility characteristic of virion DNA was present in the pre-drug serum (Fig. 2B, lane 1). This virion DNA species disappeared from the serum quickly upon ACV treatment, but other WHV DNA species, more heterogeneous and faster-migrating, persisted in these sera. We considered the diverse array of WHV DNAs detected in the sera of CW612 as a composite of two subpopulations (denoted Pl and Ml in Fig. 2). The PI DNA forms migrated more slowly in the agarose gels than a single-stranded linear WHV DNA of genomic length (designated SS); the PI species thus had the electrophoretic characteristics of a plus-strand replicative intermediate DNA. Similarly the MI DNA forms migrated faster than SS and had the characteristics of denatured minus-strand replicative intermediate DNA. The PI and MI DNAs are isolated normally only from tissues that contain immature cytoplasmic core particles. In the CW612 sera, the MI forms appeared to migrate slightly faster in successive bleeds (Fig. 2B, lanes 2–4). Neither PI nor MI DNA forms were radiolabeled in the EPA (Fig. 2A, lanes 2–4). However, the lack of WHV DNA synthesis was not due to an inhibition in the EPA resulting from ACV triphosphate present in the serum samples (see Materials and Methods). A more reasonable assumption was that in CW612, the serum PI and MI DNAs were synthesised by the normal reverse transcription reaction from the pregenome template within nucleocapsids. Thus, it seemed probable that viral DNA polymerase molecules were present in these sera and were functional in the pregenome encapsidation, minus-strand DNA priming, and elongation reactions. Rather than the viral DNA polymerase rendered intrinsically inactive by the ACV, it seemed more plausible that the PI and MI DNAs were no longer functional primer-templates. Since incorporation of ACV-monophosphate into a nascent DNA strand leads to chain termination [Furman et al., 1980], the PI and MI forms, present only in the drug phase sera of CW612, are probably DNAs arrested in synthesis by ACV-mediated chain termination. These results suggested that both in vitro and in vivo the WHV DNA polymerase was unable to excise the incorporated and chain-terminating ACV monophosphate by a 3′ exonucleolytic editing reaction and thereby enable DNA synthesis to resume.

The serum titre of WHV in an infected woodchuck was a variable parameter, presumably determined by the rate of virus secretion from the liver and the rate of clearance from the circulation. Quantitation of the EPA data for CW601 and CW612 using serial dilutions of a pre-drug serum (bleed 2) from each animal indicated that the half-life for WHV particles in woodchuck blood was 2 to 3 days. This value accords well with similar estimates made for HBV particles [Drouet et al., 1975]. Additionally, all of the serum samples were assayed for the viral surface antigen. Only minor changes in the levels of antigenaemia were detected (data not shown); these changes occurred with all four woodchucks and without correlation to the ACV treatment period. This observation indicated that WHV transcription, translation, surface antigen morphogenesis, and secretion were probably all unaffected in this ACV protocol.

Viral DNA From Untreated and ACV-Treated Liver

Frozen liver was available from the two woodchucks that died during this study (see Materials and Methods). CW657 had never received ACV or other antiviral drugs; CW612 died 26 days into the ACV treatment. Samples of both livers were extracted by two different but related protocols for analysis of the WHV DNA forms present. The two extraction procedures were both developed to selectively recover the small polyomavirus DNA in infected cells from the excess, high molecular weight mouse DNA [Hirt, 1967]. In the more conventional method, the hepadnaviral CCC DNA species are isolated in the Hirt supernatant fraction, while the replicative forms from within nucleocapsids are not recovered because of their covalent attachment to viral polymerase protein [Zoulim and Seeger, 1994; Weber et al., 1994]. The viral CCC DNA derived from nuclear minichromosomes was isolated both as SC and nicked, OC forms [Newbold et al., 1995]. The second formulation of Hirt's selective extraction protocol is rarely used, but we find it to be ideally suited for application to hepadnavirus-infected cells. In this method the extract is first digested with protease (pronase or proteinase K) prior to precipitation with 1.0 M NaCl; with this addition to the basic protocol, the high molecular weight woodchuck DNA still pelleted selectively, while the viral

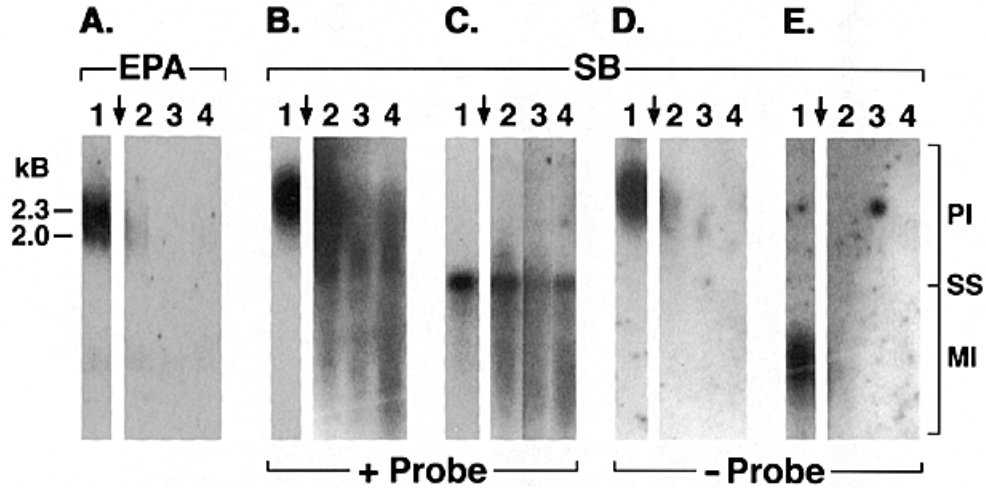

Fig. 2. Endogenous polymerase activity (EPA) and Southern blot (SB) analysis of sera from woodchuck CW612. In all panels, **lanes 1, 2, 3,** and **4** correspond to bleeds 1, 3, 4, and 5 respectively, in Figure 1. CW612 died suddenly 3 days after the serum in lanes 4 was drawn and following 26 days of ACV treatment. The arrow schematically separated the pre-drug bleed in lane 1 from the three samples taken after the ACV dosing began. **B** and **D** are duplicate gels of the native DNA preparations, separately hybridised with plus-strand (B) and minus-strand (D) riboprobes. **C** and **E** portray DNA samples identical with those in B and D that were exposed to thermal denaturation prior to electrophoresis; C and E were separately hybridised with plus- and minus-strand riboprobes, respectively. The mobility of the full-length, single-stranded WHV DNA is marked SS. The PI and MI designations identify the mobilities for the plus- and minus-strand replicative intermediate DNAs. Duplex DNA size markers (in kilobase pairs) are indicated beside the gels and define the direction of electrophoresis.

replicative intermediates that were released from core particles and no longer covalently bound to the viral polymerase were all recovered with the CCC DNA in the supernatant. The DNA preparations were electrophoresed directly and after exposure to thermal denaturation, then blotted and hybridised with both plus- and minus-strand WHV riboprobes to identify the SC, OC, PI, SS, and MI viral DNA species.

As expected, the standard Hirt protocol led to the selective extraction of the two species characteristic of hepadnaviral CCC DNA. The SC form migrated more rapidly in the agarose gel, and a relaxed OC DNA electrophoresed more slowly; both species were present, however, at comparable concentrations in the livers of CW657 and CW612 (Fig. 3, lanes C1 and C3; Table II). When these DNA preparations were exposed to thermal denaturation prior to electrophoresis, the SC form renatured [Vinograd et al., 1968] and migrated with the same mobility as in the native samples (Fig. 3, lanes C1 and C2; lanes C3 and C4). However, the OC DNA was denatured stably by the heat treatment to produce the two faster-migrating species designated SSa and SSb. These latter species of WHV DNA have been shown to represent the linear and circular denaturation products, respectively, expected from an OC DNA that contained a single random break in one DNA strand (Xin and Newbold, unpublished data).

In addition to the OC and SC DNAs, the modified Hirt extraction recovered the heterogeneous PI and MI forms that are present in viral nucleocapsids as intermediates in the reverse transcription reactions. The most abundant viral DNAs in the liver of (the untreated) CW657 were the PI and MI forms (Fig. 3, lane A1). The PI species exhibited the characteristics of a native intermediate of plus-strand DNA synthesis. It was selectively detected by a minus-strand probe (Fig. 3, lane B1), and when electrophoresed after exposure to a high temperature, was stably denatured to a full-length minus-strand DNA (species SS in Fig. 3A, lane A2) and an array of incomplete plus-strand DNAs (Fig. 3, lane 2B) with mobility similar to MI. In contrast, the MI form behaved like an intermediate in minus-strand DNA synthesis that was already fully denatured from its viral RNA template. It was not detected by the minus-strand probe (Fig. 3, lane 1) and its electrophoretic mobility (faster than SS) was unaffected by the denaturation regimen (Fig. 3, lane A2). The viral DNA profile from the liver of the ACV-treated CW612 was strikingly different (Fig. 3, lane A3). The PI DNA form was almost absent (Fig. 3, compare lanes A1 and A3); phosphoimaging revealed a sevenfold lower concentration of PI in this woodchuck compared to CW657 (Table II). Moreover, this low level of PI was detected only by the plus-strand probe (Fig. 3, compare lanes A3 and B3) and when denatured before electrophoresis yielded a full-length minus-strand DNA (species SS in Fig. 3, lane A4) without detectable short plus-strand DNA (Fig. 3, lane B4). These data indicated that the PI species from CW612 was composed of completed minus-strand DNA duplexed exclusively with short plus-strand DNA of variable length. The MI species was present in CW612 at a slightly lower concentration than in CW657 (Table 2), but its average mobility was increased (Fig. 3, compare lanes A1 and A3). This DNA species was unaffected by the denaturation treatment (Fig. 3, lane A4), was not detected by the minus-strand probe (Fig. 3, lanes B3 and B4) and seemed in character just like the MI

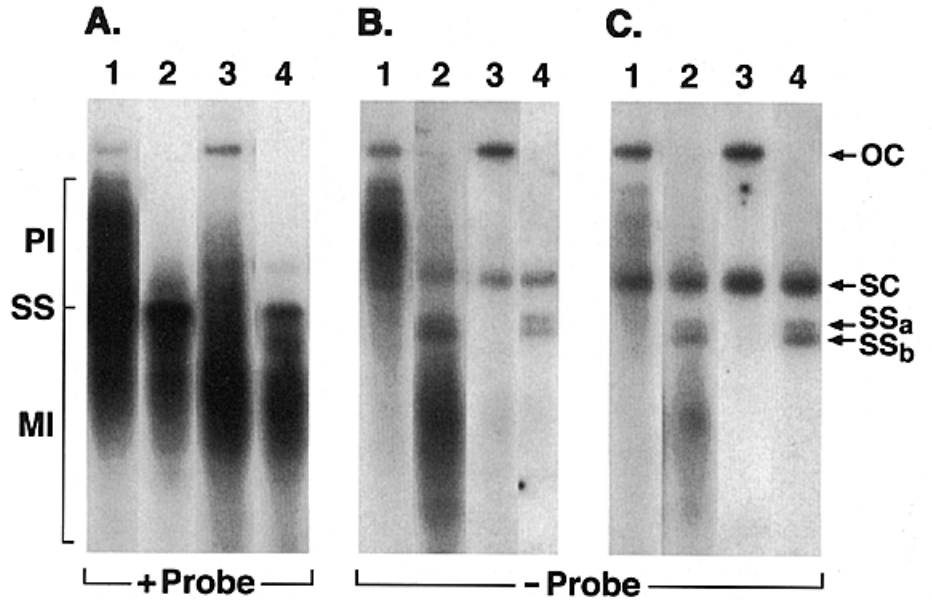

Fig. 3. WHV DNA species in the livers of CW657 and ACV-treated CW612. **A** and **B** are duplicate gels showing DNA samples isolated by the modified Hirt protocol (see Materials and Methods) and separately hybridised to plus- and minus-strand riboprobes, respectively; the WHV DNAs include the SC and OC forms (from the viral minichromosome), and the PI, SS, and MI species as replicative intermediate DNAs (from immature nucleocapsids). **C** shows similar DNA samples extracted by the standard Hirt protocol. Each panel contains paired and duplicate DNA samples from CW657 **(lanes 1, 2)** and CW612 **(lanes 3, 4);** the samples in lanes 2 and 4 were exposed to thermal denaturation prior to electrophoresis. The SS_a and SS_b species derive by denaturation from the OC DNA and are single-stranded linear (SSa) and circular (SSb) molecules.

TABLE II. Viral Minichromosome and Replicative Intermediate DNA Species From Untreated and ACV Treated Liver

	Genome equivalents per hepatocyte					
	Minichromosome			Replicative intermediates		
	OC	SC	Total	PI	MI	Total
CW657 (untreated)	6	15	21	104	443	547
CW612 (ACV)	7	12	19	14	253	267

species in CW657 but with a higher prevalence of shorter minus-strand DNAs.

The apparent bias for incomplete, minus-strand DNAs in the liver of the ACV-treated woodchuck was emphasised by a more detailed dissection of the mobility profiles of the PI and MI forms obtained from both CW657 and CW612. Phosphoimages were obtained by scanning Southern blots of these liver DNAs after probing with a plus-sense WHV riboprobe. The computer-generated images were divided into eight equal sections (Fig. 4), spanning a range of mobilities from just greater than the OC species and including the smallest MI forms. OC DNA was omitted from the analysis since it was derived from CCC DNA nicked artifactually during the isolation procedure. In the liver of CW657, roughly 70% of the hybridised plus-strand probe identified a symmetrically fractionated population of PI and MI forms centered at the mobility of SS DNA (Table III, fractions 3, 4, and 5). The corresponding zone for the liver DNA of CW612 represented a similar proportion of the minus-strand DNA, but was an asymmetric population of intermediate DNAs markedly skewed to the higher mobility. For the two liver DNA preparations shown in Figure 4, the ratio of the WHV DNAs present in each cognate fraction is listed in Table 3, confirming the bias for the MI species in the liver from the ACV-treated woodchuck.

Similarity of the WHV DNA in the Serum and Liver of the ACV-Treated CW612

The PI and MI DNAs present in the sera of CW612 after the onset of ACV dosing are displayed in Figure 2, lanes B2–4. The effects of thermal denaturation and the strand specificity for these species (Fig. 2C–E) were remarkably similar to the analogous forms detected in the liver (Fig. 3, lanes A3, A4, B3, and B4). The last serum sample prepared from CW612 was drawn 3 days prior to the woodchuck's death and, except for the absence of the SC and OC species the electrophoretic profile of this DNA (shown in Fig. 2, lane B4, and in Fig. 5, lane B1) resembled that present in the liver tissue just 3 days later. This correlative observation strongly suggested that the heterogeneous array of PI and MI species detected in the serum of CW612 had derived

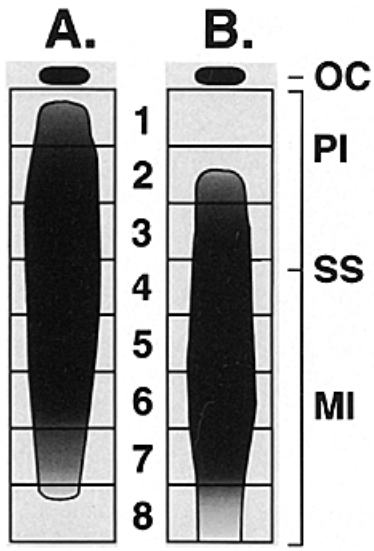

Fig. 4. Schematic for the phosphoimaging analysis of the WHV DNA recovered from two infected woodchucks; **A,** the untreated CW657, and **B,** the ACV-treated CW612. Southern blots of DNA samples identical with those shown in Figure 3A, **lanes 1** and **3,** were detected by a phosphoimager and the scans divided into the eight zones as depicted. The quantification of the WHV DNA in each zone is listed in Table III. The OC, PI, SS, and MI designations are as per Figure 3.

TABLE III. Quantification of Electrophoretic Fractions of WHV DNA Intermediates From Untreated and ACV-Treated Livers

Fraction	A (CW657:untreated)[a]	B (CW612:ACV)[a]	A/B
1	3.7	1.0	3.7
2	13.6	2.6	5.2
3	21.2	10.3	2.1
4	29.8	21.9	1.4
5	21.6	35.4	0.6
6	7.3	19.0	0.4
7	2.0	6.7	0.3
8	1.0	3.0	0.3

[a]Expressed as the percentage of total viral minus-strand DNA in liver for each fraction (see Fig. 4).

from identical intermediates in the liver of this ACV-treated woodchuck.

MI DNAs in the Serum of CW612 are Contained in Particles Essentially Identical With Virions

To determine if the PI and MI DNA forms in the serum of CW612 were present as naked core particles or, like virions, as nucleocapsids associated with a lipoprotein envelope, sera were fractionated by equilibrium density gradient centrifugation in buoyant CsC1. Serum from the initial bleed (i.e., pre-drug) and the final bleed (i.e., on ACV) were used in this analysis. Nucleic acids in the CsC1 gradient fractions were extracted and subjected to agarose gel electrophoresis, and WHV DNA was detected by the Southern blot assay with a plus-strand riboprobe. For the pre-treatment serum (Fig. 5A), WHV DNA with the mobility characteristic of virion DNA was recovered in five fractions at the buoyant density expected (1.225 g/ml) for virion particles [Summers, 1981]. Minor amounts of faster-migrating PI DNAs were detected in these same fractions as well as the original input serum (Fig. 5, lane A1). An additional minor population of viral DNA, barely detected in the input serum, was also concentrated to these fractions; this SS species had the mobility of a full-length linear minus-strand DNA (Fig. 5A).

The entire array of PI, SS, and MI DNAs present in the final serum from the ACV-treated CW612 (Fig. 5, lane B1) were recovered in the CsC1 gradient with the density distribution typical for mature virions (Fig. 5B fractions 17–21); no WHV DNA was detected elsewhere in this gradient. In this serum, the PI, SS, and MI species were evidently contained within particles having a buoyant density similar to virions. Thus, they were presumed to have the composition of enveloped nucleocapsids and to have entered the circulation by secretion from the liver using the normal pathway for enveloped WHV particles.

DISCUSSION

The administration of ACV to three naturally infected, WHV-carrier woodchucks resulted in three distinct phenotypic outcomes; only viraemia and surface antigenaemia were monitored in each woodchuck. The drug treatment did not affect the levels of surface antigen, but inhibited viraemia in each case. The three viraemic phenotypes were remarkably different. In one animal, the circulating virus was stably reduced, whereas it was transiently re-elevated for 2–3 weeks during the treatment phase in a second woodchuck. The third serum profile was the most provocative. Normal viraemia was rapidly inhibited by ACV in woodchuck CW612, only to be replaced in the circulation by virion-like particles containing atypical and more heterogeneous WHV DNAs; these latter viral DNA species had the character of replicative intermediates truncated in their synthesis by ACV-mediated chain termination. When drug dosing ceased, viraemia was rapidly restored to pre-drug levels or, transiently, to an even higher titre. Thus, this small study paralleled the clinical experience of ACV in HBV-infected patients, probably reflecting in part the marginal efficacy of ACV at this dose. However, it seems clear that at least some mammals (including man) possess the requisite enzymology in their hepatocytes to appropriately phosphorylate ACV sufficient for significant inhibition of hepadnavirus DNA synthesis. Pharmacological manipulations or modifications that could augment the efficacy of ACV would probably find useful clinical application [Fiume et al., 1989].

The variable responses to ACV in this study were probably largely idiosyncratic and due to normal physiological variation between animals and within the same woodchuck over time. Possible host variables include 1) anatomy and pharmacology for the liver and/or extrahepatic sites of WHV replication; 2) metabolism of ACV, especially variations in 5′-nucleotidase and transphosphorylase activities; 3) catabolism of ACV. Why all three

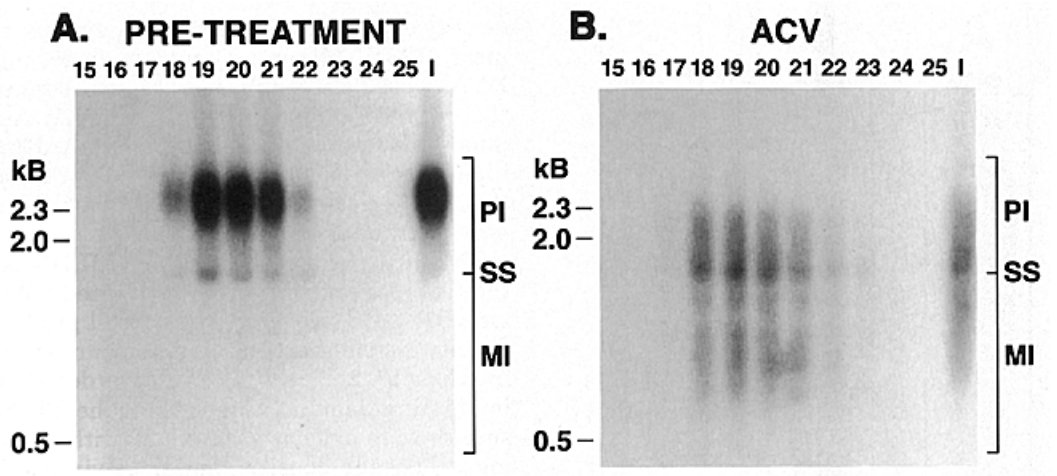

Fig. 5. Density gradient fractionation and Southern blot analysis of the sera of CW612 in CsCl buoyant for virions. **A** is a pre-treatment serum, from bleed 1 (see Fig. 1) and corresponding to the sample analysed in **lanes 1** in Figure 2. **B** is from bleed 5 (see Fig. 1) and is the same sample analysed in **lanes 4** in Figure 2. The gradient fraction numbers are indicated. Each gradient yielded 35 fractions, with increasing number reflecting decreasing density. **Lane 1** contains the DNA extracted from a sample of the serum used as input for each CsCl gradient. Both blots were hybridised with a plus-strand WHV riboprobe. Duplex DNA size markers (in kilobase pairs) are shown for both blots. The PI, SS, and MI species are as defined in Figure 2.

drug-treated woodchucks did not exhibit the same presence of aberrant, truncated replicative DNAs, as found in the serum of CW612, remains more problematic. Extremely short, drug-arrested MI DNAs may have been present in the sera within virion-like particles, but not recovered and detected by the plus-strand probe. Alternatively, very short MI forms in nucleocapsids might have been unable to proceed with membrane envelopment at the endoplasmic reticulum and thus never enter the secretory pathway of the hepatocyte. Finally, the serum profile for CW612 might have been correlated in some way with the pathology of a fatty liver as noted at autopsy in this woodchuck.

The return of viraemia immediately upon conclusion of the ACV treatment requires the persistence of a population of viral CCC DNAs that are fully transcriptionally competent. The stability of the viral CCC DNA was not measured directly in any of the three drug-treated woodchucks in this study. However, the finding of approximately 20 copies of this species per hepatocyte in one animal (CW612) that received ACV for 26 days and one (untreated) sham-treated woodchuck (CW657) suggests that WHV CCC DNA is at least a moderately stable molecule whose half-life in vivo is probably 20–30 days or longer. It was recently reported in the DHBV system that the half-life of viral CCC DNA was 3–5 days in congenitally infected primary duck hepatocytes treated in vitro with bromo-deoxyribouridine (BudR) [Civitico and Locarnini, 1994]. Another study with ducks, in contrast, inferred that DHBV CCC DNA in vivo was probably as stable as host DNA and likely turned over only at the death of the infected hepatocyte [Fourel et al., 1994].

The serendipitous availability of the liver of the ACV-treated CW612, coupled with sera from the same animal, provided a novel data set that seems to be in conflict with the dominant hypothesis for virion morphogenesis. The WHV DNA species in both liver and drug-phase sera of the CW612 were remarkably similar. This observation stands in sharp contrast to many analogous comparisons made for hepadnavirus systems in the absence of antiviral treatments. From such data, it had been proposed that envelopment of hepadnaviral nucleocapsids at the endoplasmic reticulum requires the development of a conformational change or morphogenetic signal which marked the surface of the viral nucleocapsid; the hypothetical signal was undefined but necessarily linked to a stage in plus-strand DNA synthesis [Summers and Mason, 1982]. Several consequences are implied from this hypothesis for a chronic hepadnaviral infection treated with an effective inhibitor of viral DNA synthesis: 1) by the normal process of secretion, the liver would become depleted of nucleocapsids containing PI forms in which the signal for envelopment developed; 2) core particles containing drug-arrested MI species and lacking the morphogenetic signal would be retained in the cytoplasm of the infected hepatocyte; 3) at the instant that viral DNA synthesis became inhibited, the liver would contain a normal number and size distribution of MI DNAs, but thereafter, retain and accumulate a much larger number of nucleocapsids that contain truncated MI species with shorter average length; and 4) the serum would initially contain truncated PI forms that would be cleared at the normal rate, and not replaced once the pool of PI DNAs in the liver was depleted; truncated MI DNAs would of course never appear in the serum as enveloped nucleocapsids. The DNA samples from the drug-treated CW612 adhered quite well to some of these predictions. Compared to the profile from a normal liver, exemplified by CW657, the PI forms were considerably less abundant in the liver of CW612 after 26 days of dosing with ACV. Furthermore, the size distribution of the MI forms was clearly shifted to truncated and less completely synthe-

sised molecules. This observation strongly indicated that most of the MI DNAs in the liver of CW612 had been initiated de novo after the onset of the drug treatment and nevertheless still attained an average chain length of several hundred nucleotides. Not in full accord with the hypothesis was the rather modest level of MI DNA in the liver of CW612 as compared to CW657; the calculated value of 253 MI molecules per hepatocyte did not represent the accumulation of an excess of MI DNAs over CW657. Assuming that ACV did not affect the rate of encapsidation of the pregenomic RNA, or the envelopment and secretion process, it remains a formal possibility that core particles containing drug-arrested MI forms were degraded intracellularly. The finding of virion-like particles in the serum of CW612, containing apparently the identical WHV MI DNAs, suggested an alternate fate for these core particles: egress via the secretory pathway from the hepatocyte into the circulation. Additionally, if the half-life for these virion-like particles containing drug-truncated MI DNAs is, like normal virions, 2–3 days, then the levels of these particles in the penultimate and final bleeds also indicate an ongoing production and infusion of these particles from the liver throughout the course of treatment. Thus, secretion of these apparently enveloped particles containing MI species seems to be a stable feature of CW612.

The secretion of unusual MI DNA species within enveloped nucleocapsids as inferred in this study does not render untenable the concept underlying the morphogenetic signal hypothesis. Under normal circumstances a selective advantage may well be gained for the hepadnavirus by linking genome maturation, nucleocapsid morphogenesis, and virion secretion. However, in a situation where mature nucleocapsids are depressed or absent, and core particles containing immature MI DNAs are being accumulated in the cytoplasm, their secretion as enveloped virion-like particles may be a default exigency to prevent a potentially toxic storage condition within infected cells. These data do require an accommodation with the morphogenetic signal hypothesis as previously articulated. Other studies have also detected hepadnaviral SS DNA species in virion particles. HBV SS DNA has been reported both in patient's sera [Scotto et al., 1985] and in the culture fluids of transfected cells [Sells et al., 1987; Yaginuma et al., 1987]. Also, in this study, the presence of a small fraction of WHV DNA was detected in virion particles as SS molecules in the pre-drug sera of CW612 (Fig. 5A); we do not know whether these DNA molecules have yet initiated plus-strand DNA synthesis. Since so much of the analysis supporting the hypothesis of the essential morphogenetic signal has derived from the DHBV system, it is also possible that differences between the ortho- and avi-hepadnaviridae account for these observations. Finally, when levels of viraemia are used to evaluate prospective antiviral agents, the secretion of arrested replicative intermediates must be taken into account for the correct assessment of drug efficacy.

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